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Chemical profiling with cytokine stimulating investigations of *Sutherlandia frutescens* L. R. (*Br.*) (Fabaceae)

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ABSTRACT

Sutherlandia frutescens, one of the medicinal plants of southern Africa, has been widely used to boost the immune system by various ethnic groups. This study aims to provide initial scientific evidence for *in vitro* immune modulating activities of extracts of *S. frutescens* on cytokines, including interleukins 4, 6, 8, 10, 12p70 and TNF produced by the HL60 cell lines, as well as to identify possible compounds present therein. This will assist in guiding further studies to an active compound. The chemical profile of extracts, fractions and compounds was determined using high performance liquid chromatography coupled to mass spectrometry. Results from the initial *in vitro* experiments conducted indicated that extracts from *S. frutescens* possessed immune modulating as well as anti-inflammatory activities. This *in vitro* study showed that an ethanolic extract appeared to recruit the various inflammatory cytokines to the site of infection upon stimulation with phorbol 12-myristate 13-acetate, where essentially the non-polar compounds present in the ethanol extract contributed to most of the activity observed for this extract.

1. Introduction

Immunodeficiency is a condition in which the immune system, normally a first line of defence against unwanted pathogens, is unable to contain infectious diseases. As a result, an immune-compromised individual will often succumb to more severe infections of longer duration than normal, causing the body to become chronically emaciated (Farlex, 2012).

Various plants have been used for a variety of medicinal purposes, and have contributed significantly to the development of major medical drugs that are still in use today (Simpson and Ogorzaly, 2001). Several immunomodulatory effects have been attributed to medicinal plants (Actor and Dasgupta, 2003; Spelman et al., 2006), with great potential for their use as pharmacological agents to treat immune disorders and modulate immune-related pathogenesis (Clement-Kruzel et al., 2008). Among these are *Echinacea* spp., *Astragalus membranaceus*, *Zingiber officinale*, *Panax ginseng* and *Sambucus nigra* (Dewick, 2002; Spelman et al., 2006; Vukovic, 2004). Many scientific investigations have been conducted on these plants to demonstrate their immune boosting effects (Burger et al., 1997; Raduner et al., 2006; Spelman et al., 2006). Botanicals have also been used to suppress an over reactive immune system such as *Siphonochilus aethiopicus* (publication in process) and the drug cyclosporine initially isolated from the fungus *Tolypocladium inflatum* (Borel, 2002).

Southern Africa is rich in plant diversity and the use of traditional medicines is widespread and promoted by the Ministry of Health. The South African Department of Health has recommended two herbal remedies (*Hypoxis hemerocallidea* and *Sutherlandia frutescens*) for the management of many ailments, including cancer and for patients with human immunodeficiency virus (HIV) infection (Mills et al., 2005a).

S. frutescens, one of the most widely used indigenous plants of southern Africa, is commonly found in the South West and Northern Cape provinces (Fig. 1). It belongs to the family Fabaceae (pea and bean or pod-bearing family). There are five *Sutherlandia* species

Abbreviations: ACN, acetonitrile; BD, Beckton Dickinson; CBA, cytometric bead arrays; CP, chemically pure grade; ECACC, European collection of cell culture; Ech, *Echinacea*; ESI$^{-/+}$, electrospray ionisation (negative or positive mode); EtOH, ethanol; FBS, foetal bovine serum; HIV/AIDS, human immunodeficiency virus/acquired immunodeficiency syndrome; HL60, human leukaemia cell line; HPLC–MS, high performance liquid chromatography–mass spectrometry; IL, interleukin; IR, immune response; LPS, lipopolysaccharide; NMR, nuclear magnetic resonance; PDA, photo diode array; PMA, phorbol 12-myristate 13-acetate; PRR, pattern recognition receptors; Sa, *Siphonochilus aethiopicus*; SANBI, South African National Biodiversity Institute; SQD, single quadruple detector; TLC, thin layer chromatography; TNF, tumour necrosis factor.

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namely, *S. frutescens*, *Sutherlandia microphylla*, *Sutherlandia montana*, *Sutherlandia tomentosa* and *Sutherlandia humilis*. The genus took its name from James Sutherland, first director of the Edingburgh botanic garden (Duncan, 2009). The term 'sutherlandia' refers to a shrubby plant that is known by many different names including cancer bush, umnwele (Xhosa), Insiswa (Zulu), and Phetola (Sotho) (Jackson, 1990). Various tribes have used this plant for medicinal purposes, particularly chronic diseases, including cancer (Van Wyk and Albrecht, 2008). This study was undertaken to provide a scientific basis to support the use of this plant amongst traditional healers for immune-compromised and cancer-infected individuals.

Literature reports have also indicated that extracts from *S. frutescens* can also be used in the treatment of HIV/AIDS (Harnett et al., 2005; Mills et al., 2005b), cancer (Chinkwo, 2005; Stander et al., 2007; Tai et al., 2004) as well as to treat diabetes (Chadwick et al., 2007; Sia, 2004). The anti-cancer and anti-viral properties of extracts from *S. frutescens* have been attributed to the presence of L-canavanine (Green, 1988). In addition, extracts have also been shown to have anti-bacterial, anti-oxidant (Katerere and Eloff, 2005), anti-inflammatory (Kundu et al., 2005) and anti-mutagenic (Reid et al., 2006) activities. Ngcobo (2008) showed that low concentrations of extracts of *S. frutescens* on a primary culture of T cells could stimulate immune cells.

A number of various *in vivo* and *in vitro* techniques exist to study immune modulation of natural products for example the *in vivo* use of flow cytometry and immunohistochemistry in a standard primate toxicology evaluation for the diagnosis of potential immunomodulatory effects (Lappin and Black, 2003) or the *in vitro* stimulation of cytokine production in lipopolysaccharide (LPS)-induced cells (Clement-Kruzel et al., 2008; Raduner et al., 2006; Spelman et al., 2006; Zidek et al., 2009). Numerous alternative techniques are available to assess the immune modulation of natural products (Haddad et al., 2005; McKay and Blumberg, 2007; Spelman et al., 2006; Wagner, 1990). The technique of using stimulated cytokine expression in various immune cells has been widely used. Spelman et al. (2006) suggested that the immune modulating effects of the botanical medicines reviewed may be due to cytokine modulation. Additionally, the review article from Zidek et al. (2009) has concluded that cytokine and anti-cytokine immunotherapies have proved to provide valuable therapeutic effects. As such, modulation of cytokine secretion may offer alternative approaches in the treatment of a variety of diseases, especially in immune-compromised patients (Spelman et al., 2006). Novel therapeutic strategies targeting the cytokine network are needed to enhance the effectiveness of current immunotherapeutic procedures (Zidek et al., 2009). Cytokines are key mediators in an immune response (IR), which are produced by cells of the immune system in response to a stimulus and help to recruit or orchestrate other immune cells to the site of infection (Lydyard et al., 2004; O'Gorman and Donnenberg, 2008; Parkin and Cohen, 2001). However, the effectiveness of immunotherapies may vary since, the biological effects of several cytokines are often overlapping and individual cytokines possess multiple regulatory functions. The enhancement of efficacy of immunotherapeutic treatments may therefore lead only through more complex and novel strategies (Zidek et al., 2009). It is therefore important that such studies should assay a variety of cytokines as well as assess various other factors involved in the immune response. Follow up *in vivo* evaluations should be coupled to such research activities.

This research aims to show preliminary *in vitro* immune modulation activities of extracts prepared from fresh and dried *S. frutescens* plant material, using an HL60 model system with stimulated cytokine quantification; as well as to identify possible active compounds in the extracts using high performance liquid chromatography–mass spectrometry (HPLC–MS) analysis. HPLC is a powerful and versatile analytical technique used to obtain chromatographic profiles of plant extracts (Holme and Peck, 1998; Hostettmann et al., 2010; Wolfender, 2009); while MS, coupled to HPLC, allows for information on the identity and structure of the components therein. In the drug development process, MS has been used for lead compound discovery, structural analysis, synthetic development, combinatorial chemistry, pharmacokinetics and drug metabolism (Pavia et al., 2009). While a number of various review articles have contrasted the limitations as well as the potential of HPLC–MS (Goetzinger et al., 2004; Marston and Hostettmann, 2009; Wolfender, 2009), it nevertheless remains one of the most widely and frequently used techniques in the study of natural products, particularly where separation, identification and quantification of compounds present in a given sample is concerned (Marston and Hostettmann, 2009). A study done by Avula et al. (2010) also employed the use of HPLC–MS to confirm the presence of the previously isolated compounds (Fu et al., 2008, 2009) found in *S. frutescens*; this technique has therefore been employed in this study as well. As such, the reported outcomes of this paper will serve to guide future researchers to pay more attention to the 'active regions' of the HPLC–MS chromatograms to ultimately isolate the active compound(s).

2. Materials and methods

2.1. Chemicals

CP grade solvents were purchased from Merck and distilled before use. Solid phase extraction C-18 cartridges (Supelco 140 ml PP tubes) were purchased from Sigma Aldrich. Distilled water was used for all procedures. HPLC-grade solvents were purchased from Microsep

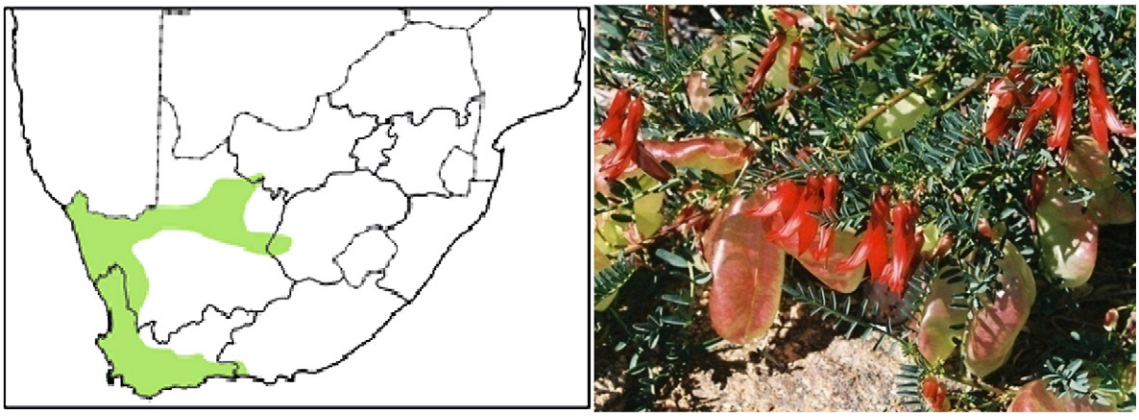


Fig. 1. Geographical distribution of *Sutherlandia* species throughout South Africa on the left (sahealthinfo.org) and on the right *S. frutescens* growing in the wild at Goegap Nature Reserve, Springbok, Northern Cape (photo taken by N. Harding).

(Romil pure chemistry solvents). RPMI-1640, foetal bovine serum (FBS) and L-glutamine were purchased from Whitehead Scientific. Gentamicin, phorbol 12-myristate 13-acetate (PMA) and etoposide were purchased from Sigma Aldrich. The Cytometric Bead Array™ Human Inflammation Cytometric Bead array (CBA) kit (551811) was purchased from Beckton Dickinson (BD) Biosciences and analysed using BD Biosciences' equipment and software.

2.2. Plant collection

S. frutescens (L.) R. B.r. plant material was collected from a cultivation site in the Free State, South Africa (GPS coordinates: 29° 6.774′ S; 25° 24.305′ E). Identification of the specimen deposited at the South African National Biodiversity Institute (SANBI), Tshwane was confirmed (SANBI voucher specimen number: 428679).

2.3. Extract preparations

Fresh leaves (488 g) were boiled in 12.5 l of H_2O for 1 h with occasional stirring. The suspension was then filtered and freeze-dried. The aqueous extract from the fresh leaves was labelled extract A (47.8 g; 9.8% yield). The air-dried leaves (200 g) were extracted with ethanol (EtOH; 1.6 l of 96% v/v) at room temperature, stirred, left overnight and then filtered and evaporated to give extract B (11.17 g; 55.9% yield). Another 200 g of the air-dried leaves was boiled in 2.0 l of H_2O for 1 h with occasional stirring. This solution was filtered and freeze-dried (extract C, 33.72 g; 16.9% yield).

2.4. Preparation of fractions

The ethanol extract B was fractionated using solid phase cartridges. 10 g of extract B (EtOH) was dissolved in 20 ml H_2O and loaded on the activated C-18 (Supelco 140 ml PP tubes, Sigma Aldrich) cartridge. Different solvents were used to generate the fractions: 200 ml 100% H_2O, 160 ml 20% MeOH, 110 ml 40% MeOH, 160 ml 60% MeOH, 160 ml 80% MeOH, 200 ml 100% MeOH and 200 ml 100% acetonitrile (ACN). The resulting fractions were evaporated and freeze dried and combined to give fractions I to III according to TLC profiles.

2.5. Quantification of cytokines with CBA

The HL60 cell line was obtained from the European collection of cell culture (ECACC) and maintained in suspension at 37 °C in 5% carbon dioxide (CO_2) and 100% relative humidity in RPMI-1640 supplemented with 5% foetal bovine serum (FBS), 2 mM L-glutamine and 50 μg/ml gentamicin. Cells were counted and inoculated in a 96-well microtitre plate at plating densities of 7000 to 10 000 cells per well and incubated for 24 h. Test samples (50 μl) were added to specific wells at a concentration of 25 μg/ml and incubated for 48 h. Phorbol 12-myristate 13-acetate (PMA) (12.5 ng/ml) was used as a cytokine stimulant and added to the wells at 42 h, where needed, and placed in the incubator for the remaining 6 h. Cells without drug addition served as the control, while the blank contained complete cell culture media without cells. Ethanolic preparations of *Echinacea* spp. and *S. aethiopicus* were used as positive (immune boosting) and negative (immune suppressant) controls respectively, and were also tested with and without PMA. After 48 h of incubation, the plate was removed and centrifuged for 2 min at 1000 *g*, supernatants (50 μl) from each well were removed and placed in Eppendorf tubes separately.

The IL12p70, TNF, IL10, IL6, IL1β and IL8 cytokines were detected using the human inflammation Cytometric Bead Array (CBA) kit (551811; BD Biosciences). Tests were performed according to the manufacturer's instructions available online. The six bead populations are resolved in a red channel of a BD FACSCalibur flow cytometer. For each set of experiments, a standard curve was generated. The results were expressed as pg/ml and then analysed for their relative expression (control versus treated samples). The lower limit for detection for each cytokine was determined as 10 pg/ml. In order to simplify results from the assays a numbering system as outlined in Table 2 was used.

2.6. Statistical analysis

All determinations were done in quadruplicate, and the results were reported as mean ± standard deviation (sd). Graphs were plotted using Origin version 6.0 (Microcal Software, Inc.).

2.7. HPLC MS analysis

For each extract and fraction, approximately 25 mg was weighed out into a vial and 2 ml of HPLC-grade solvent (methanol, acetonitrile or water depending on the solubility of the sample) was added. This mixture was then put in an ultrasonic bath for 10 min and filtered through Acrodisc GHP syringe filters, before being placed into 2 ml HPLC vials. The various samples were analysed using a WATERS 2695 HPLC separation module. Two Atlantis T3 columns (10×250 mm, 5 μ particle size) connected in series, were used for the separation. UV–VIS detection was done on a WATERS PDA scanning from 200 to 600 nm. The mobile phase used was 0.1% (v/v) formic acid in water (A), methanol (B) and acetonitrile (C). The ratio of mobile phase prepared is given in Table 1 below. Additionally, mass spectrometry detection was performed using a WATERS SQD scanning from 100 to 1200 *m/z* with polarity (+/−) switching with a scan time of 0.20 s. The operating conditions in the ESI source were as follows: source temperature, 150 °C; desolvation temperature, 450 °C; capillary voltage, 3.00 kV; cone voltage, 30.0 V. Gas flow (N_2): desolvation, 800 l/h; cone gas, 10 l/h.

2.8. Isolation of compounds

Sutherlandioside B was a kind gift from Prof. Albrecht, while sutherlandiosides A and D (Fig. 2) were isolated from the plant extract following the procedure described by Fu et al. (2008). Only a small amount of the compounds was isolated and therefore no further biological activity was able to be conducted on them. For structure elucidation of the compounds, NMR spectroscopy was performed using a 600 MHz Varian NMR.

Accurate mass analysis was performed on a WATERS Synapt G1 UPLC-TOF-MS system. Two column types were used namely, WATERS Acquity CSH C18 (150×2.1 mm, particle size: 1.7 μ) for the polar compounds and WATERS Acquity HSS T3 (150×2.1 mm, particle size: 1.8 μ) for the more polar compounds.

3. Results and discussion

The extracts and fractions of *S. frutescens* were analysed for their activities on the release of cytokines with PMA as a co-stimulant on

Table 1
Gradient timetable for the HPLC-SQD method used.

Time (min)	Flow rate (ml/min)	% A FA H_2O	% B MeOH	% C ACN
0	0.3	95	5	0
4	0.3	95	5	0
5	0.3	75	25	0
27	0.3	55	45	0
30	0.3	42	58	0
70	0.3	22	78	0
80	0.3	12	88	0
85	0.3	12	88	0
90	0.3	0	100	0
100	0.3	0	100	0
107	0.3	0	0	100
113	0.3	0	0	100
115	0.3	95	5	0

Table 2
Numerical values corresponding to the various samples used in the cytokine quantification assay.

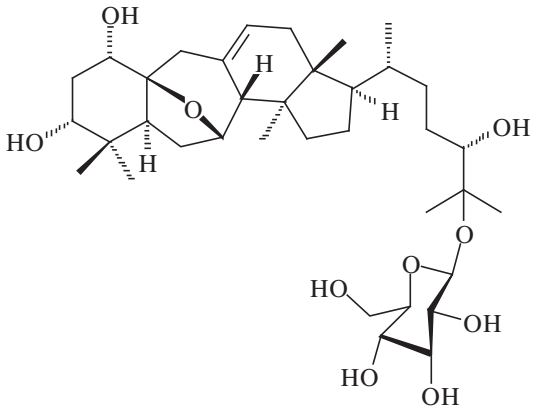
Number	Sample
1	Control (Ctrl.)
2	PMA (12.5 ng/ml)
3	Extract A (fresh leaves)
4	A + PMA
5	Extract B (EtOH)
6	B + PMA
7	Extract C (dry leaves)
8	C + PMA
9	Fraction I
10	I + PMA
11	Fraction II
12	II + PMA
13	Fraction III
14	III + PMA
15	*Echinacea* (Ech)
16	Ech + PMA
17	*Siphonochilus aethiopicus* (Sa)
18	Sa + PMA

HL60 cells. For immune cells to work effectively they need to be recruited to the sites of inflammation and appropriately activated. This is achieved by cellular receptors and associated cytokines that bind to these receptors (Lydyard et al., 2004; O'Gorman and Donnenberg, 2008; Parkin and Cohen, 2001). The cytokines IL1β, IL6, IL10, IL12p70, IL8 and TNF, used in this assay, were selected due to their role in various pro- and anti-inflammatory actions involved in the first step of the IR i.e. inflammation.

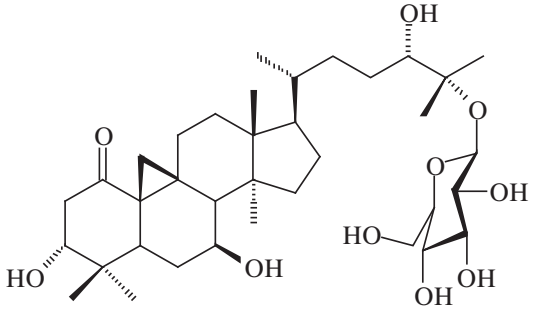
From the results of the six cytokines analysed, in the above *in vitro* experiment, no effect was found on IL1β, IL6, IL10 and IL12p70 (as depicted in Fig. 3), therefore the following section will be focussed on the change in TNF and IL8 only (Fig. 4a and b, respectively). In this experiment three controls were used namely, the blank control (number 1 in Figs. 3, 4a and b), a positive control (*Echinacea* extract, number 15 in Figs. 3, 4a and b) and a negative control (*S. aethiopicus* extract, number 17 in Figs. 3, 4a and b). No release of any of the six cytokines (i.e., initiation of an immune response) was observed when all the extracts and fractions of *S. frutescens* were applied to the HL60 cells without the co-stimulation of PMA (represented by odd numbers in Figs. 3, 4a and b). When PMA (number 2 in Fig. 4a and b) was added to the cell culture system alone, there was a higher production of IL8 (4551.95 ± 410.85 pg/ml) and an improvement in the amount of TNF (129.69 ± 19.21 pg/ml) being released into the supernatant when compared to the blank control (which had a concentration of 50.83 ± 7.74 pg/ml).

Specifically fraction III from the ethanol extract; together with PMA (number 14 in Fig. 4a and b), contributed to a marked increased release of the TNF and IL8 cytokines (229.45 ± 13.89 for TNF and 5967.93 ± 226.86 pg/ml for IL8). The aqueous extracts and fractions I and II, together with PMA, displayed a marked decrease of IL8 being released (concentrations ranging from about 2909 to 3260 pg/ml). As is apparent in Fig. 3, it was observed that the *S. frutescens* extracts did not affect subsequent release of IL1β, IL6, IL10 and IL12p70 in the culture system, relative to PMA, after a 48 h incubation period (concentrations varied from 3 to 20 pg/ml).

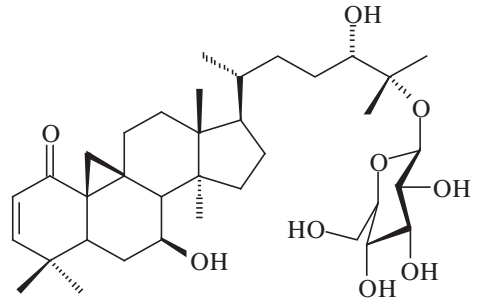
To correlate the activities found with the *in vitro* cytokine experiment, all extracts were then analysed using HPLC–MS to obtain their chromatographic profiles. The resulting profiles of extracts A, B and C in ESI^- (electrospray ionisation negative mode) are shown in Fig. 5, while those for fractions I, II and III in ESI^+ are given in Fig. 6. The cycloartane glycosidic compounds — sutherlandioside A, B and D — were isolated from an extract of *S. frutescens* and identified in the HPLC–MS profiles of the resulting extracts and fractions. In the chromatographic profile of extract B they were found to elute at 57.24, 43.51 and 59.25 min, respectively (labelled 1, 2 and 3 in Figs. 5 and 6). A higher amount of these compounds can be found in fraction II relative to the other fractions (Fig. 6). From the chromatographic profiles of the fractions it can be extrapolated that the majority of the polar compounds can be found in fraction I and the aqueous extracts (extracts A and B, Figs. 5 and 6), which could be mainly made up of flavonol glycosidic compounds similar to the ones isolated by Fu et al. (2009), due to their similar interaction with the column used as well as their similar mass fragmentation patterns obtained from HPLC–MS analysis. A structural analysis, which included MS interpretation, UV analysis and a 3D structure evaluation, was conducted on fraction I to predict the possible compounds present in order to provide some justification that these compounds might be the flavonol glycosides. A flavonoid detection technique using ferric (iii) chloride hexahydrate showed the presence of a higher concentration of flavonoids in fraction I. The cycloartane glycosidic compounds (which were isolated, identified and evaluated on the HPLC–MS) were found in higher quantities in fraction II as well as extract C, while the non-polar compounds appeared in fraction III. The method described by Avula et al. (2010) showed the



Sutherlandioside A (compound number 1)



Sutherlandioside B (compound number 2)



Sutherlandioside D (compound number 3)

Fig. 2. Structures of sutherlandiosides A, B and D are respectively referred to as compounds 1, 2 and 3.

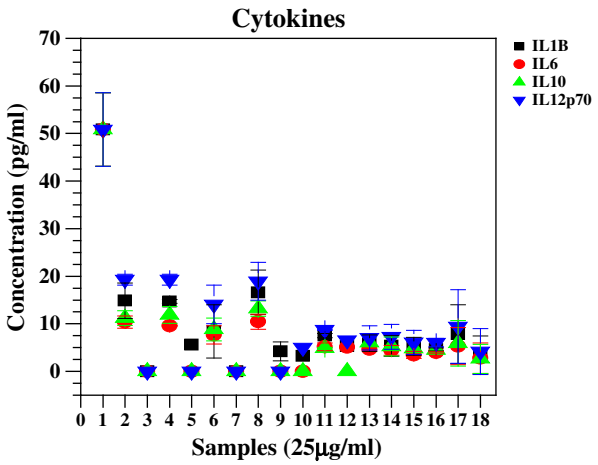


Fig. 3. Release of IL1β, IL6, IL10 and IL12p70 cytokines for the blank control, PMA (at 12.5 ng/ml), extracts A to B, fractions I to III, positive and negative controls at 25 µg/ml for a 48 h incubation period. For statistical analysis n = 4.

cycloartane glycosides (sutherlandiosides A to D, isolated by Fu et al., 2008) were found to elute from around 24 to 30 min (the more intermediate polarity region of their chromatographic profile). By evaluating the isolated sutherlandioside A, B and C compounds on the HPLC–MS these results concur with the above method that the cycloartane glycosides were also found in the intermediate polarity region (from about 35 to 60 min) from the chromatographic profiles obtained of the extracts and fractions (Figs. 5 and 6). The surrounding compounds in this region also possess mass fragmentation patterns similar to these compounds, thus proposing that this region mainly contains the sutherlandiosides and other related compounds of intermediate polarity. No biological activity was conducted on the isolated compounds as there were insufficient amounts.

PMA (acting as a mitogen) was used as an initiator of the increased release of cytokines into the culture supernatant, which was taken as a measure of immune response initiation potential (Fig. 4a and b, sample number 2). This arrangement resulted in a co-stimulated release of TNF and IL8 into the culture supernatant well above that of the baseline levels found with the blank control.

As PMA forms part of the lipopolysaccharide (LPS) of gram negative bacterial cells, it can be recognised by pattern recognition receptors (PRR) present on the target cells used in this study (Lydyard et al., 2004). As such, the expression of TNF is stimulated by LPS interaction with PRR on the HL60 cell line and subsequently IL8 is activated by the release of TNF. TNF activation is consequently coupled with programmed cell death (apoptosis) initiated by infection with a particular pathogen and also coincides with the release of inflammatory mediators which stimulate recruitment of other immune cells to the site of infection (Lydyard et al., 2004). The release of IL8 is believed to help the immune system to increase chemotaxis for neutrophils (as well as for T cells and basophils) at the site of inflammation (Lydyard et al., 2004; Parkin and Cohen, 2001).

In contrast to the PMA alone, the effect of fraction III from the ethanol extract (containing mostly the non-polar compounds as shown in Fig. 6) on the target cells, with the co-stimulation of PMA, exhibited a greater release of TNF and IL8 into the culture supernatant. In essence, this observed effect, together with the up-regulated release of specific cytokines, could be taken to mimic a possible *in vivo* situation of a bacterial infection in a given host, being combated by the release of appropriate cytokines. A study conducted by Katerere and Eloff (2005) provided concurrent results that anti-staphylococcal activity resided in the non-polar fractions. Not much focus has been given to the isolation and identification of the non-polar compounds found in *Sutherlandia* species as most of the focus has been on the flavonol- and the cycloartane glycosidic compounds; therefore further research into the isolation and identification of pure compounds from the non-polar region should be conducted.

The decreased influence of *S. frutescens* extracts on the release of IL8, together with the co-stimulation by PMA in the *in vitro* cell culture system, was mainly found in the aqueous extracts. According to the method described by Avula et al. (2010) it was found that the flavonol glycosides (sutherlandins A to D, isolated by Fu et al., 2009) were found to elute from around 8 to 12 min (the polar region of their chromatographic profile). The chromatographic profiles of the extracts and fractions obtained here, together with the low interaction between polar compounds and the column stationary phase as well as resulting mass fragmentation patterns of the compounds eluting in the polar region of the extracts (from about 15 to 25 min), collectively suggest that these compounds could be those of the flavonol glycosides, or related compounds found in *Sutherlandia* species. Therefore, the observed effect of the aqueous extracts plus fraction I could

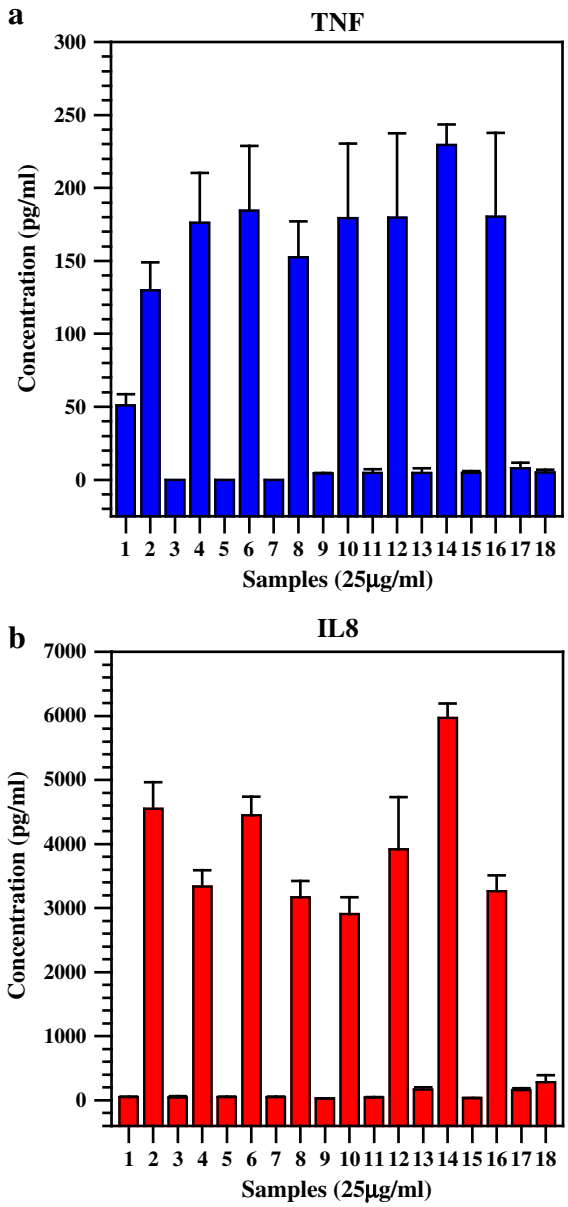


Fig. 4. Release of TNF (a) and IL8 (b) for the blank control, PMA (at 12.5 ng/ml), extracts A to B, fractions I to III, positive and negative controls at 25 µg/ml for a 48 h incubation period. For statistical analysis n = 4.

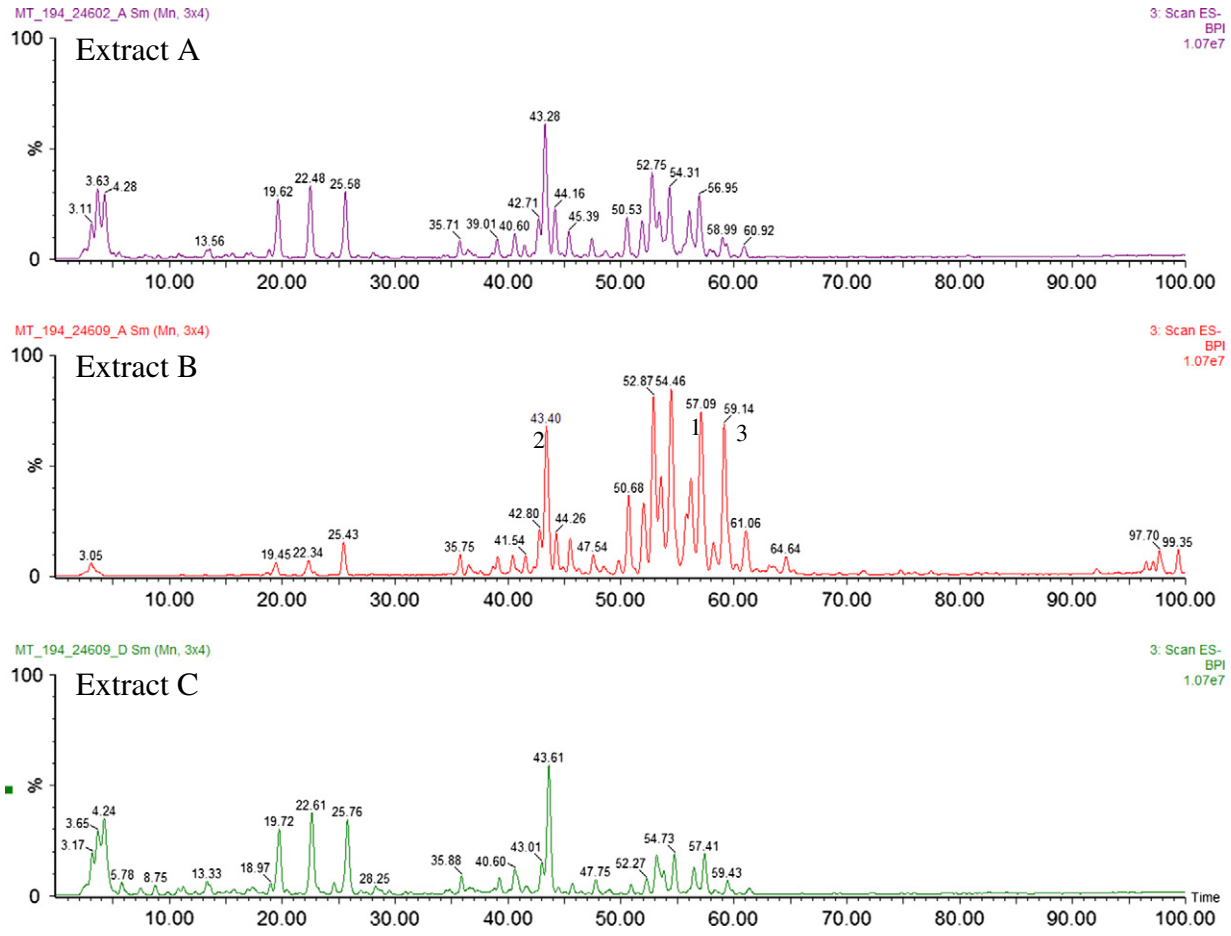


Fig. 5. The HPLC ESI⁻ chromatograms for extracts A, B and C. 1, 2 and 3 in the chemical profile of extract B indicates the presence of sutherlandiosides A, B and D, respectively.

possibly be related to the presence of flavonol glycosides and their polar interactions with cells of the *in vitro* cell culture system.

As stated above, IL8 plays a role in inflammation and therefore the reduction of IL8 found with the aqueous extracts could possibly be related to the anti-inflammatory properties of *S. frutescens*. Fernandes et al. (2004) have indicated that a hot water extract of *S. frutescens* possessed superoxide, as well as hydrogen peroxide scavenging activities. This could account for some of the anti-inflammatory properties that have been described in the literature. In addition, while anti-inflammatory properties associated with various medicinal plant extracts have been explained, at least in part, by their anti-oxidant activities (Gali-Muhtasib et al., 1999; Gerritsen et al., 1995; Lindsey et al., 2002; Schinella et al., 2002), extracts from *S. frutescens* have also been demonstrated to have anti-oxidant activity in reducing free radical cations (Tai et al., 2004). In addition, Kundu et al. (2005) have shown that a methanolic extract of *S. frutescens* possessed anti-inflammatory activity by inhibiting 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced COX-2 expression in both *in vitro* and *in vivo* models of carcinogenesis. Collectively, all these findings have provided mechanistic support to the effect that plant extracts, via their active constituents connected with anti-inflammatory properties, also demonstrate anti-tumour promoting potential as well.

A noteworthy observation from this *in vitro* experiment was that for immune boosting effects, an ethanolic preparation would have been more beneficial. Similarly, for an anti-inflammatory response, an aqueous preparation could have achieved better results. Further research accordingly needs to be conducted in this direction. An ethanolic extract of *S. frutescens* was shown to exhibit no significant anti-oxidant effects (Tai et al., 2004). However, aqueous extracts were shown to possess significant anti-inflammatory and anti-oxidant effects (Fernandes et al., 2004; Katerere and Eloff, 2005; Kundu et al., 2005). A study done by Ngcobo (2008) showed that high concentrations of aqueous as well as ethanol extracts of *S. frutescens*, could reduce the production of IL1β and TNFα, which are regarded as being helpful in fighting muscle wasting associated with cancer and HIV/AIDS patients. Anti-inflammatory agents have also been shown to exhibit chemo-preventative activity (Surh et al., 2001; Surh, 2002); therefore, the anti-inflammatory properties attributed to the aqueous extracts of *S. frutescens* could possibly be used in a chemo-preventative setting as well.

The differential increase in IL8, together with the co-stimulation by PMA, of the non-polar compounds found in the ethanol extract, could also have contributed to possible immune stimulating effects. For this reason, preparations of *Sutherlandia* seem to exhibit many attributes that could be related to the complex nature of compounds present in the raw plant material. The different varieties of compounds present in the various extracts of *S. frutescens*, therefore provide initial findings that support its various traditional uses. However, further *in vitro* and *in vivo* studies would be required to confirm this supposition. The aqueous extracts displayed a similar effect as the positive control (*Echinacea*) (Burger et al., 1997; Raduner et al., 2006; Sharma et al., 2010; Spelman et al., 2006; Vukovic, 2004), while the negative control (*S. aethiopicus*) displayed a complete

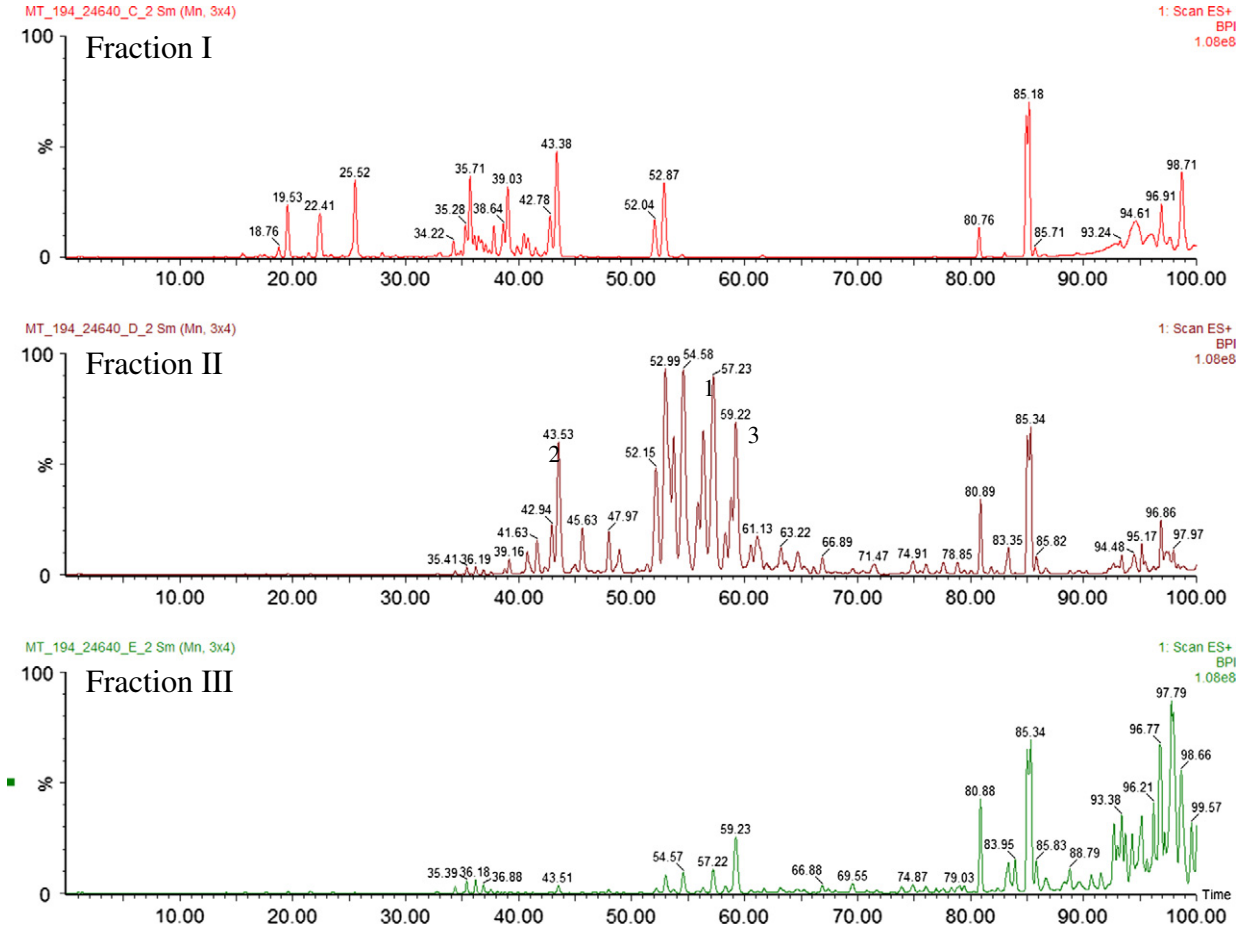


Fig. 6. HPLC ESI$^+$ chromatograms for fractions I, II and III.

suppression of IL8 and TNF, even with the co-stimulation of PMA, thus demonstrating immunosuppressive potential of extracts from this plant. *S. aethiopicus* has been known to have immunosuppressive characteristics by suppressing the production of certain cytokines needed for the immune response, such as IL8 (publication in process). The plant contains an active furanoterpenoid compound that is known to inhibit the nuclear transcription factor NF-κB involved in the regulation of many pro-inflammatory factors.

4. Conclusion

The results from the above *in vitro* experiment have provided initial evidence that the non-polar compounds, present in the ethanol extract of *S. frutescens*, could suggestively amplify the release of specific, immune-modulating cytokines, particularly by cells already stimulated by a pathogenic micro-organism, such as a gram negative bacterium. These results have also indicated that the cultured cells need to be in a condition where they were intrinsically releasing certain cytokines, so that when an a-polar fraction of an ethanolic preparation was subsequently added, such as to HL60 cells, they were able to up regulate and release more of the particular cytokines involved in the IR. This could be of great interest for the treatment of diseases that specifically attack the immune system, such as HIV/AIDS and cancer, resulting in a more rapid expulsion of the invader micro-organism, with subsequent limitation of the likelihood of further spread of the infectious agent to surrounding cells. HPLC–MS analysis also made it possible to determine that the non-polar compounds were more helpful at stimulating the *in vitro* immune system, while the aqueous extracts (more polar compounds) were found to precipitate an anti-inflammatory type response. Overall, from the initial findings of the cytokine assay, particular attention needs to be given to the correct choice of solvent used for an extract preparation of *S. frutescens*, in order to select for a specific biological effect. Although this research had a limitation of testing the roles of pure compounds isolated from the *S. frutescens*, this manuscript acts as an insightful perspective to the active regions of the extracts of *S. frutescens* for further isolation of pure compounds. At the moment, the research group is busy in isolation and identification of pure compounds from extracts of *S. frutescens*, especially focussing on the non-polar region (the active section). The results of this study will be reported soon. Supplementary investigations into isolation and identification of the non-polar compounds (present in the non-polar region of the ethanol extract) could be of great value in future studies of this kind, especially where various in depth *in vivo* immune stimulating models and animal studies are concerned.

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